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Defects in Mitochondrial Protein Synthesis and Respiratory Chain Activity Segregate with the tRNA$^{Leu(UUR)}$ Mutation Associated with Mitochondrial Myopathy, Encephalopathy, Lactic Acidosis, and Strokelike Episodes

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Cytoplasts from two unrelated patients with MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis, and strokelike episodes) harboring an A→G transition at nucleotide position 3243 in the tRNA$^{Leu(UUR)}$ gene of the mitochondrial genome were fused with human cells lacking endogenous mitochondrial DNA (mtDNA) (ρ^0 cells). Selected cybrid lines, containing <15 or ≥95% mutated genomes, were examined for differences in genetic, biochemical, and morphological characteristics. Cybrids containing ≥95% mutant mtDNA, but not those containing normal mtDNA, exhibited decreases in the rates of synthesis and in the steady-state levels of the mitochondrial translation products. In addition, NADH dehydrogenase subunit 1 (ND 1) exhibited a slightly altered mobility on polyacrylamide gel electrophoresis. The mutation also correlated with a severe respiratory chain deficiency. A small but consistent increase in the steady-state levels of an RNA transcript corresponding to 16S rRNA + tRNA$^{Leu(UUR)}$ + ND 1 genes was detected. However, there was no evidence of major errors in processing of the heavy-strand-encoded transcripts or of altered steady-state levels or ratios of mitochondrial rRNAs or mRNAs. These results provide evidence for a direct relationship between the tRNA$^{Leu(UUR)}$ mutation and the pathogenesis of this mitochondrial disease.

The mitochondrial encephalomyopathies, a clinically, morphologically, and biochemically diverse group of disorders, have recently begun to be described genetically. In the past 3 years, specific mitochondrial DNA (mtDNA) point mutations have been found to be associated with several specific syndromes (14, 18, 19, 25, 37, 41, 43, 45, 46). One of these, MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis, and strokelike episodes), is characterized by lactic acidosis, episodic vomiting, seizures, migrainelike headaches, short stature, and recurrent cerebral insults resembling strokes and causing hemiparesis, hemianopia, or cortical blindness (36). Recently, a point mutation in the tRNA$^{Leu(UUR)}$ gene of the human mitochondrial genome has been described (14, 25, 41) and has been found to be associated with the majority of patients with the clinical features of MELAS (8, 13, 14, 16, 25). MELAS patients harboring the mutation are heteroplasmic, with the proportion of mutated mtDNAs usually exceeding 80% in muscle (8, 13).

A point mutation in the structural gene for a tRNA may be expected to result in a deficiency in translation. This has recently been shown for another mitochondrial encephalomyopathy, MERRF (myoclonic epilepsy and ragged-red fibers) (6), which is caused by a point mutation in the mitochondrial tRNA^{Lys} gene (37, 45). However, inhibition of translation due to a mutated tRNA gene may occur at several levels. The mutation may directly affect the mitochondrial tRNA function in translation or, alternatively, may affect recognition of the tRNA by an enzyme not directly involved in translation, such as the enzymes which process the large polycistronic transcripts of the mtDNA. Thus, the mutation could result in both qualitative and quantitative changes in tRNA$^{Leu(UUR)}$, as well as in 16S rRNA and NADH dehydrogenase subunit 1 (ND 1) mRNA, which flank this tRNA in the primary transcript.

The MELAS-3243 mutation also occurs in an important regulatory control region that is responsible for the specific termination of transcription at the end of the rRNA genes in vitro (7, 27). The mutation has recently been shown to impair in vitro the proper termination of transcription at the end of the 16S rRNA gene, by preventing proper binding of a protein factor that promotes this termination (17). If termination does not occur, rRNA transcription should continue on the heavy strand, which also encodes 12 of the 13 mitochondrial polypeptides. Since the rRNA genes are normally transcribed at a rate 15 to 20 times higher than the rate for protein-coding genes (11), the mutation could alter the normal ratios of rRNA to mRNA, which in turn would affect proper expression of some or all of the mitochondrial mRNAs.

We describe here the use of a cell culture system for the molecular genetic analysis of the MELAS-3243 mutation. This system is based on the isolation of human cell lines that completely lack mtDNA (ρ^0 cell lines) and the ability to repopulate these cells with exogenous mitochondria (22). These ρ^0 cells were repopulated with mitochondria from two unrelated patients with the MELAS-3243 mutation, creating a number of clonal cybrid cell lines that contained from 0 to 100% mutant mtDNAs. Using genetic, biochemical, and morphological methods, we have found that the MELAS-3243 mutant genotype segregates with quantitative deficiencies in protein synthesis, respiratory chain activity, and cell growth.

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MATERIALS AND METHODS

Patients. Patient 1 is patient 4 in Table 4 of Ciafaloni et al. (8). She was a 15-year-old girl with delayed milestones, short stature, recurrent vomiting, weakness, pigmentary retinopathy, and cardiomyopathy, but with no strokelike episodes. Family history was compatible with maternal inheritance. Lactate was increased in blood and cerebrospinal fluid, and muscle biopsy showed numerous ragged-red fibers. She fulfills all the clinical criteria for MELAS except for strokelike episodes. Patient 2 is patient 7 in Table 1 of Ciafaloni et al. (8) and is unrelated to patient 1. She was an 11-year-old girl with short stature, exercise intolerance, and recent decline in school performance. She has had a few strokelike episodes with hemianopia, hemiparesis, and nuclear magnetic resonance imaging evidence of cortical infarcts. Family history was compatible with maternal inheritance. Lactic acid was increased in blood and cerebrospinal fluid, and she had calcifications of the basal ganglia. She fulfills the clinical criteria for MELAS. Muscle biopsy showed numerous ragged-red fibers, and biochemical studies of isolated mitochondria showed complex I deficiency. Both patients had the MELAS-3243 mutation.

Cell culture. The 143B and 143B206 cell lines, which have been described previously (22), were grown in Dulbecco's modified Eagle medium containing 4.5 mg of glucose per ml and 110 μg of pyruvate per ml (DMEM) supplemented with 50 μg of uridine per ml, 100 μg of 5-bromodeoxyuridine (BrdU) per ml, and 5% fetal bovine serum (FBS). Fibroblasts of patient 1 (a gift from A. Pestronk) and myoblasts of patient 2 (a gift from A. F. Miranda) were grown in DMEM supplemented with 50 μg of uridine per ml and 15% FBS. Transformation of the 143B206 cell line with patient mitochondria by cytoplast fusion was done as described previously (22). Cybrids were replated 24 h after fusion in DMEM supplemented with 100 μg of BrdU per ml and 5% dialyzed FBS (selective medium). The medium was changed to DMEM supplemented with 50 μg of uridine per ml, 100 μg of BrdU per ml, and 5% FBS (nonselective medium) 9 to 11 days after fusion. Cybrids were replated at low cell density in nonselective medium in order to pick cell clones 12 to 32 days after fusion. In one instance, the cybrids of patient 1 were plated at low density in selective medium. Individual cell clones were picked by using a glass cylinder 9 to 14 days later. All lines were subsequently grown in DMEM supplemented with 50 μg of uridine per ml and 5% FBS. All cell lines described in this report tested negative for the presence of mycoplasma by DNA transfer hybridization analyses, using as a probe plasmid pSP64-129, which contains the insert of M13Mh129 (12).

DNA analyses. Total DNA was extracted from exponentially growing cells according to established protocols (9). DNA was analyzed for the MELAS mutation (the A→G mutation creates a new *Hae*III site at position 3243) (mtDNA numbering according to Anderson et al. [2]) by polymerase chain reaction (PCR), using primers corresponding to the light-strand positions 3116 to 3134 and to the heavy-strand positions 3353 to 3333 for 25 cycles of 1 min at 94°C, 1 min at 55°C, and 0.75 min at 72°C. To this PCR product was added 25 pmol of each primer, 10 μCi of [α-^{32}P]dATP (3,000 Ci/mmol), and 2.5 U of *Taq* polymerase, and the mixture was incubated for 2 min at 94°C, 1 min at 55°C, and 12 min at 72°C. The resulting product was digested with the restriction enzyme *Hae*III and electrophoresed through a 12% nondenaturing polyacrylamide gel. The DNA restriction fragments were quantitated using a Betascope 603 blot analyzer (Betagen, Inc.). The ratio of mtDNA to nuclear DNA was determined as described previously (3, 33). For nuclear DNA variable-number tandem repeat analysis, total DNA was digested with *Pvu*II or *Hin*dIII and, after electrophoresis and transfer, was hybridized with plasmid pYNH24 (35). The mtDNA was analyzed for the presence or absence of a *Hae*III restriction site at position 16517 by PCR amplification of the appropriate region of the mitochondrial genome (primers spanning either nucleotides [nt] 16000 to 175 or nt 15812 to 720), followed by *Hae*III restriction digestion of the PCR product and agarose or acrylamide gel electrophoretic analysis of the resulting restriction fragments. For the analysis shown in Fig. 1, 10 μCi of [α-^{32}P]dATP (3,000 Ci/mmol) was included in the PCR reaction and the restriction fragments were visualized by autoradiography.

RNA analyses. Total RNA was isolated from exponentially growing cells (42) and electrophoresed through 1.2% agarose-formaldehyde gels (28), transferred to Zeta-Probe membranes (Bio-Rad), and hybridized according to the manufacturer's suggested protocols. The following probes for RNA analyses were labeled by random priming (10): pHFβA-1, a plasmid containing the human cytoplasmic β-actin gene (15); an mtDNA fragment corresponding to a portion of the ND 5 gene (a PCR product extending from positions 13949 to 14276); and two *Mse*I mtDNA fragments corresponding to the tRNA$^{Leu(UUR)}$ gene (positions 3232 to 3264 and 3265 to 3291). The latter were obtained by *Mse*I restriction digestion of the insert of mp8.M11 (mtDNA positions 3063 to 3659 [21]) followed by gel isolation and ligation of the appropriate fragments. Strand-specific probes of the following mtDNA clones were obtained by extension of the universal M13 primer according to the method of Sucov et al. (39): mp9.M9 and mp8.M9 (mtDNA nt 1 to 739), which hybridize to 12S rRNA and 7S RNA, respectively; mp19.BR54 (nt 3659 to 4122), which hybridizes with ND 1; mp18.XB66 (nt 7658 to 8287), which hybridizes with cytochrome *c* oxidase subunit II (COX II); and mp19.XS6.2 (nt 2441 to 2954), which hybridizes with 16S rRNA (21). RNA hybridization signals were quantitated with a Betascope 603 blot analyzer.

Analysis of the mitochondrial translation products. Exponentially growing transformants and 143B cells (~10^6 cells on 60-mm petri dishes) were labeled with [^{35}S]methionine (>1,000 Ci/mmol, 100 to 200 μCi/ml) for 10 to 90 min in 2 ml of methionine-free DMEM supplemented with 50 μg of uridine per ml, 100 μg of emetine per ml, and 5% dialyzed FBS as described by Chomyn et al. (6). The translation products were analyzed on 15 to 20% sodium dodecyl sulfate (SDS)-polyacrylamide gradient gels (5) and subjected to fluorography on Kodak X-Omat AR films. The assignment of the mitochondrial translation products was based on two criteria: (i) similarity of the labeling pattern to that described by Chomyn and Lai (5) and (ii) absence of each labeled polypeptide in a parallel labeling experiment performed with 143B206 cells. Equal amounts of total cellular protein, as determined by the method of Lowry et al. (29), were loaded in each lane of any single experiment. Quantitation of mtDNA-encoded polypeptides on fluorograms of appropriate density was performed with a Molecular Dynamics 300A computing densitometer.

Oxygen consumption. Oxygen consumption by intact cells was determined as described previously (22) except that 5 × 10^6 cells were resuspended in 1.65 ml of DMEM without glucose and there was no serum supplementation of the medium.

Cytochemistry and immunocytochemistry. Cells grown on glass coverslips were stained cytochemically for COX and

succinate dehydrogenase (SDH) activities and immunostained with antibodies against mtDNA-encoded COX II, ND 1, nuclear-encoded COX IV, and the lipoyl acetyltransferase (E2) portion of the pyruvate dehydrogenase complex (PDH), as described previously (30, 32). COX II and ND 1 antibodies (kind gifts of J. R. Doolittle) were polyclonal and were used at a dilution of 1:250. COX IV monoclonal antibody (34) was used at a dilution of 1:100. PDH polyclonal antibodies (40) were diluted 1:200. The coverslips were examined and photographed with a Zeiss II photomicroscope equipped with epi-illumination.

RESULTS

Construction and characterization of MELAS cybrids. To investigate the effects of the MELAS-3243 mutation on the expression of the mitochondrial genome and the respiratory function of mitochondria, mitochondria from patients with the MELAS mutation were introduced into human cells lacking endogenous mtDNA. Fibroblasts from patient 1 and myoblasts from patient 2 were enucleated, and the resulting cytoplasts were fused with an excess of cells from the human ρ^0 cell line 143B206 (22). After fusion, the cells were replated in medium lacking pyrimidines and containing BrdU. Since ρ^0 cells are thymidine kinase deficient and auxotrophic for pyrimidines because of the lack of a functional respiratory chain, only the ρ^0 cells which had fused with cytoplasts would be expected to grow. Any residual intact myoblasts and fibroblasts, as well as any ρ^0 cells which had not fused with cytoplasts or which had fused with residual intact fibroblasts and myoblasts, would not be able to grow in this medium. After 9 days for patient 1 and 11 days for patient 2, uridine was added to the growth medium, and unless noted otherwise, the cells were subsequently grown in this medium. This was done in order to eliminate any growth disadvantage of cells containing mutations affecting mitochondrial function, which is required for uridine-independent growth. There were eight independent fusion groups for each patient, and several hundred presumptive cybrids were observed in each. To pick cell clones, cybrids were replated at low cell density 12 to 25 days after fusion for patient 1 and 28 to 32 days after fusion for patient 2. In one instance, the cybrids of patient 1 were plated at low density in selective medium.

The A→G transition in MELAS at position 3243 creates an additional *Hae*III restriction site in the mtDNA, resulting, after cleavage with *Hae*III, in 97- and 72-bp fragments in the mutant, rather than the 169-bp fragment found in the wild-type mtDNA (8). By amplifying this region of the mitochondrial genome by PCR and cleaving the amplified fragment with *Hae*III, the proportion of mutant and wild-type genomes present in each sample can thus be quantitated. Total DNA was isolated from cell samples of randomly chosen clonal cell lines and analyzed for its percentage of MELAS mutation. The values observed in the cell lines derived from each patient ranged from 100% wild-type to 100% mutant mtDNA. Of 80 cell lines examined, 26% contained less than 10% mutant mtDNA and 30% contained more than 90% mutant genomes. There were no significant differences in the ranges between the two patients. Of 15 clones selected for rapid growth in the absence of pyrimidines, i.e., a medium requiring mitochondrial function, and analyzed for their mtDNA content, 7 had <10% mutant mtDNA and 3 had >90% mutant mtDNA.

From the quantitation of the proportion of mutant genomes, eight cybrid cell lines derived from patient 1 and 10 cybrid lines derived from patient 2 were chosen for further characterization (Table 1). With the exception of cybrid cell lines RN164 and RN214, all cybrid cell lines were independent; i.e., they were derived from independent cell fusions or, if derived from the same fusion group, contained vastly different percentages of mutant mitochondrial genomes. Five lines from patient 1 and 6 from patient 2 contained ≥95% mutant mitochondrial genomes. The remaining cybrid lines contained ≤1% mutated mtDNA (except for WS214, which had 12%). The latter will be referred to in this report as wild-type cell lines, although variation among human mtDNA sequences (4) precludes any one mtDNA genotype from being designated as the wild-type sequence. Included in the cell lines from patient 1 were two wild-type cell lines (WS239 and WS241) and two mutant lines (WS227 and WS240) selected for rapid growth in the absence of pyrimidines. The levels of mutant and wild-type mtDNA in each of the cybrid lines did not vary, even after 8 weeks of growth in cell culture. Analyses of the mtDNA from two cybrid cell lines that contain virtually 100% wild-type mtDNA and two that contain nearly 100% mutant genomes from each patient are shown in Fig. 1A.

The mtDNA and nuclear DNA genotypes of each of the 19 lines of this study were confirmed by examining mtDNA and nuclear DNA for restriction fragment length polymorphisms (RFLPs). As can be seen in Fig. 1B for the representative cell lines, the mtDNA of the patient 1 cybrids had the same RFLP as patient 1's blood and was different from that of 143B. The cybrids of patient 2 had the same mtDNA RFLP as patient 2's blood, which was different from that found in patient 1. However, this RFLP did not distinguish the mtDNA of patient 2 from that of 143B. The nuclear DNA of all the cybrids had the same variable-number tandem repeat

TABLE 1. Cells and cell lines used

Cell or cell line	% MELAS mutation	Analysis performed[a]					
		Protein	Cyt	Imm	O_2	Growth	RNA
Patient 1 fibroblasts	56	−	−	−	−	−	−
Patient 2 myoblasts	54	−	−	−	−	−	−
143B (ρ^+)	0	+	+	+	+	−	+
143B206 (ρ^0)	0	−	−	−	+	+	−
Wild-type cybrids							
WS214	12	+	+	+	+	−	+
WS239	0	+	+	+	+	+	−
WS241	0	+	−	−	+	−	+
RN110	0	+	−	−	+	−	−
RN131	1	+	−	−	+	−	−
RN204	0	+	+	+	+	−	+
RN236	0	+	+	+	+	+	+
Mutant cybrids							
WS176	99	+	+	+	+	+	−
WS186	97	+	+	+	+	−	−
WS216	99	+	+	+	+	−	+
WS227	96	+	+	+	+	−	+
WS240	95	+	−	−	+	+	−
RN108	98	+	−	−	+	−	−
RN141	99	+	−	−	+	−	−
RN164	98	+	+	+	+	+	+
RN186	96	+	−	−	+	−	−
RN214	97	+	−	−	+	−	−
RN223	95	+	+	−	+	−	+

[a] Protein, labeling of mitochondrial translation products; Cyt, cytochemistry for SDH and COX activity; Imm, immunocytochemistry for COX II, COX IV, ND 1, and PDH; O_2, oxygen consumption; growth, growth curves; RNA, Northern analysis. +, analysis was performed; −, analysis was not performed.

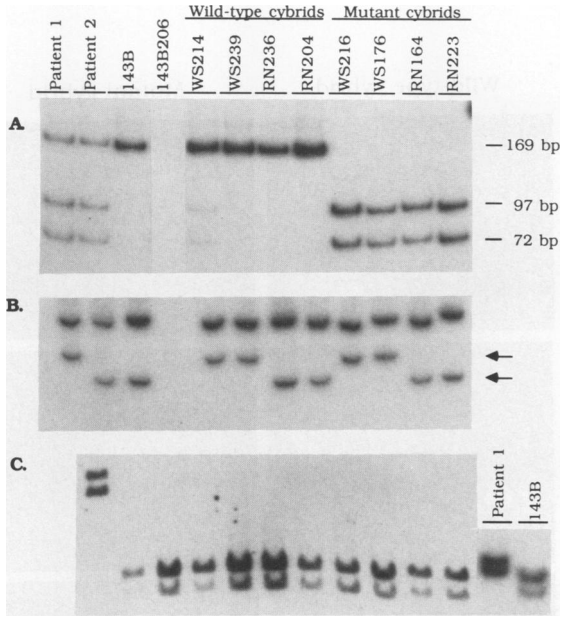

FIG. 1. Genetic characterization of parent, donor, and cybrid cells. (A) Analysis for the presence and quantity of the MELAS-3243 mutation in total DNA isolated from patient 1 fibroblasts, patient 2 myoblasts, and parent and cybrid cells, using *Hae*III digestion of a PCR-amplified fragment containing the $tRNA^{Leu(UUR)}$ gene. Sizes of the fragments are shown at the right. No analysis was performed on 143B206 cells. (B) Analysis of mitochondrial DNA polymorphisms in total DNA isolated from donor and cybrid cells and in blood from patients 1 and 2, using *Hae*III digestion of a PCR-amplified fragment between mtDNA positions 16000 and 175; an extra *Hae*III site at position 16517 distinguishes the mtDNAs of 143B and patient 2 from that of patient 1. The polymorphic fragments are indicated by arrows at the right. No analysis was performed with 143B206 cells. (C) Analysis of nuclear DNA RFLPs by DNA transfer hybridization of *Pvu*II-digested total DNA (same as in panel B) probed with the variable-number tandem repeat probe pYNH24; the probe distinguishes among all three cell types. DNA of patient 1 is not present in the first lane; the analysis of patient 1 and 143B, from a separate experiment, is shown at the right.

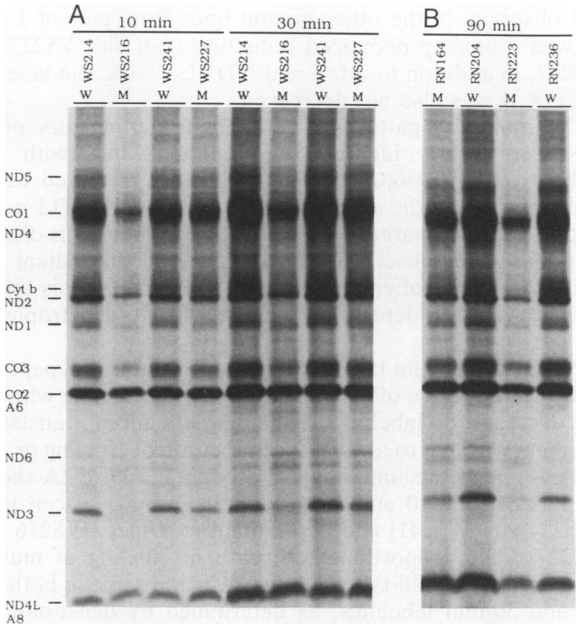

FIG. 2. Analysis of mitochondrial translation products in cybrid cell lines with and without the MELAS-3243 mutation. Fluorograms of mitochondrial translation products labeled with [^{35}S]methionine after electrophoresis through 15 to 20% SDS-polyacrylamide gradient gels are shown for the indicated mutant (M) and wild-type (W) cybrid cell lines. Mutant and wild-type cybrid cell lines derived from patient 1 (A) were labeled for 10 or 30 min in the presence of 185 μCi of [^{35}S]methionine per ml and 100 μg of emetine per ml. Equal amounts of an SDS lysate of total cellular protein (40 μg) were loaded in each lane of the gel. Mutant and wild-type cybrid cell lines derived from patient 2 (B) were labeled for 90 min in the presence of 100 μCi of [^{35}S]methionine per ml and 100 μg of emetine per ml. Equal amounts of an SDS lysate of total cellular protein (50 μg) were loaded in each lane of the gel. Cyt b, cytochrome *b*.

marker as that of their nuclear parent, 143B206, and differed from those found in both patients (Fig. 1C).

The mtDNA content of cells was determined for the majority of the cybrid cell lines and 143B by measuring the ratio of mtDNA to nuclear DNA in DNA transfer hybridization analyses of *Pvu*II-digested total DNA (3, 33) hybridized with mtDNA and with a plasmid containing an 18S rRNA gene (44). The cybrids exhibited ratios of mtDNA to nuclear DNA that were very similar to that of 143B (average ratios within each group of 0.5 to 0.6), demonstrating that the mtDNA complement of the cybrids was reestablished at a level very similar to that of the 143B cell line, in agreement with previous observations on the repopulation of the ρ^0 cells (22). No significant differences in the levels of mtDNA were observed between cybrids with or without the MELAS-3243 mutation.

Cybrids containing mutant mtDNAs exhibit a mitochondrial protein synthesis defect. All lines were examined for their pattern of mitochondrial protein synthesis by labeling cells with [^{35}S]methionine in the presence of emetine, an inhibitor of cytoplasmic protein synthesis. As can be seen in Fig. 2B, after labeling of cells for 90 min, there was a generalized decrease in the incorporation of [^{35}S]methionine into the mitochondrial-encoded polypeptides in cybrid lines with the MELAS-3243 mutation (RN164 and RN223) compared with those without the mutation (RN204 and RN236). The extent of the decrease in incorporation varied somewhat between different mutant cell lines, as can be seen between RN164 and RN223. However, a decrease in the labeling of mutant lines compared with the wild types was always observed and was typically 50 to 70%, as measured by densitometry. Some of the larger mtDNA-encoded polypeptides such as ND 2 and COX I and III were particularly affected. In contrast, the labeling of ND 6 and ND 4L + A 8 was either unchanged or increased by about 50%. While the ND 4L and A 8 genes contain the fewest number of Leu(UUR) codons (1 and 2, respectively), the decrease in labeling found in the other polypeptides did not correlate with the number or percentage of Leu(UUR) codons present in the genes coding for these proteins. This is particularly evident for ND 6, whose gene contains 14 Leu(UUR) codons, more than any other mitochondrial gene, and whose relative labeling is actually increased in the mutant cybrid cell lines. Conversely, COX III, which contains only three Leu(UUR) codons, is significantly decreased in labeling intensity.

Similar results were obtained with cybrid lines derived from patient 1. As with patient 2, the decrease in labeling varied among the 5 different mutant lines examined. Figure 2A shows the labeling patterns of two of the mutants, WS216 and WS227. The labeling pattern in WS216 was typical of

that observed in the other mutant lines from patient 1 and showed a more pronounced reduction than did WS227. In WS227, in addition to ND 6 and ND 4L + A 8, the labeling of COX II was also not decreased.

The qualitative patterns of mitochondrial translation products were nearly identical between cell lines with and without the mutation. The only difference observed was a slight increase in the electrophoretic mobility of ND 1 in the mutant lines compared with the wild-type lines. This change in mobility was observed for all but one of the mutant cell lines from both patients, although the degree of this difference appeared to depend on the conditions of electrophoresis.

We examined the labeling of mitochondrial polypeptides with shorter pulses of [^{35}S]methionine to determine whether the decrease in labeling of the mitochondrial translation products was due to a decrease in the rate of labeling or to a decrease in the accumulation of products. Figure 2A shows the labeling for 10 and 30 min of two wild-type cell lines (WS214 and WS241) and two mutant cell lines (WS216 and WS227). The proportional decrease in labeling of mutant compared with wild-type cell lines was the same in both the 10- and 30-min labelings, as determined by densitometry. These results suggest that the rate of synthesis of the mtDNA-encoded polypeptides is decreased in the cell lines harboring the MELAS-3243 mutation.

To determine whether the steady-state levels of the mitochondrial translation products were decreased, we used immunocytochemical staining of cells grown on coverslips to observe in a semiquantitative manner representative mitochondrially synthesized polypeptides. Figures 3A to D show staining of two cybrid cell lines, RN236, a wild type, and RN164, a mutant, with primary antibodies directed against PDH, ND 1, COX II, and COX IV, respectively. PDH, a nuclear-encoded, mitochondrially located complex, shows the typical discrete, particulate staining of mitochondria in the cytoplasm. No difference in staining between cells with and those without the MELAS mutation was observed, nor was this staining different from that of 143B cells (data not shown). Similar results were obtained with antibodies directed against COX IV, another nuclear-encoded polypeptide, in which both mutant and wild-type cells had similar levels of staining. However, with antibodies against either ND 1 or COX II, both mtDNA-encoded polypeptides, there was a clear decrease in the staining in cells harboring the MELAS mutation compared with the staining of wild-type cells. Similar patterns of staining were seen in six cell lines of patient 1 (four mutant and two wild type). This finding implies that there is a decrease in the steady-state levels of the mtDNA-encoded polypeptides in mutant cells, while those of nuclear-encoded polypeptides, including subunits of the respiratory chain complexes, are unchanged.

Mutant cybrids are deficient in respiration. The respiratory capacity of the cybrid cell lines was examined by determining oxygen consumption of intact cells and by cytochemical analysis of COX and SDH activities. As can be seen in Fig. 4, the rate of oxygen consumption of the mutant cybrid lines was significantly lower (mean = 1.4 ± 1.0 fmol per cell per min) than that in the wild-type cybrids (mean = 5.3 ± 0.8 fmol per cell per min), an average reduction of 74%. The respiratory capacities of the wild-type cybrids of patient 1 and 2 were, in general, very similar and similar to that of 143B, the parent of the ρ^0 cell line. The respiratory capacities of cybrids WS241 and WS239 were virtually identical to that of the other wild-type cybrids of patient 1, even though these cybrids were selected for rapid growth in the absence of

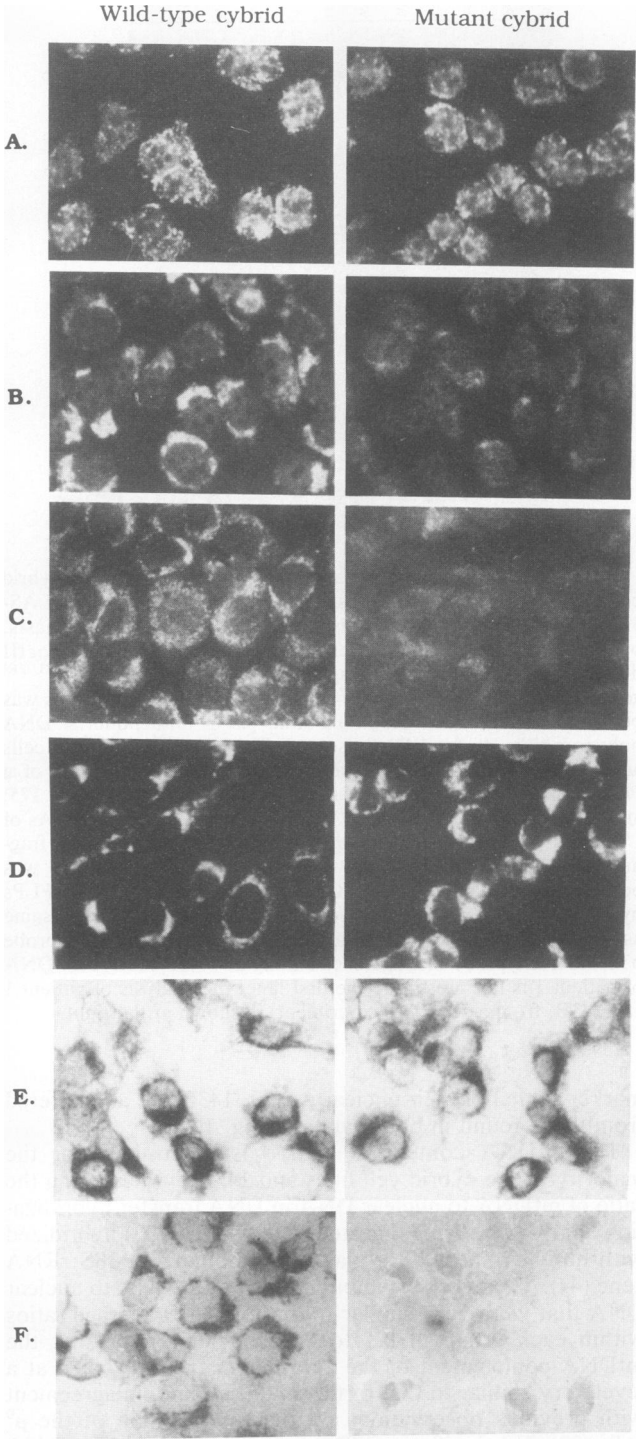

FIG. 3. Immunocytotochemistry and cytochemistry of mutant and cybrid cell lines. Wild-type (RN236) and mutant (RN164) cybrids from patient 2 were grown on coverslips in nonselective medium and analyzed by immunocytochemistry for PDH, ND 1, COX II, and COX IV (A to D, respectively) and by cytochemistry for SDH (E) and COX (F) activities. Each pair of photos comparing RN236 with RN164 was shot under identical conditions of exposure and contrast.

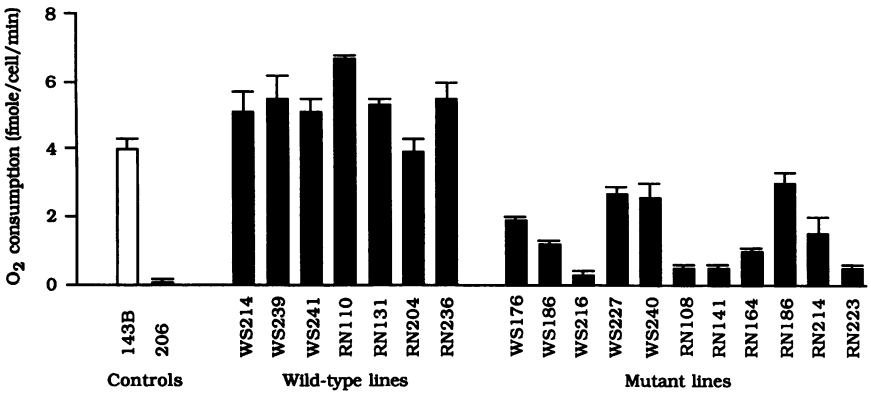

FIG. 4. Oxygen consumption. Rates of oxygen consumption per cell of 143B, 143B206, and the indicated cybrid cell lines are shown, with error bars representing 2 standard errors. The identification of each cell line is shown below the bars.

uridine, a condition requiring mitochondrial function. In contrast, in the mutant cybrid lines of patient 1 selected for rapid growth in the absence of uridine, WS240 and WS227, rates of oxygen consumption were significantly higher than those of the other mutant cybrids of patient 1 but still lower than those of the wild-type cybrids.

Cytochemical staining of the various cybrid cell lines for activity of SDH, a multisubunit enzyme of the respiratory chain whose polypeptides are all nuclear encoded, showed no significant differences between the mutant and the wild-type cell lines (Fig. 3E). However, staining for activity of COX, which is encoded by both mtDNA and nuclear DNA, showed striking differences between those lines with and without the MELAS mutation. Markedly reduced staining for COX activity was seen in the mutant compared with the wild-type cell line (Fig. 3F). A similar weak level of COX staining was observed in one of the mutant lines from patient 1 (WS176), whereas a complete absence of staining was observed in the other mutant cybrid lines analyzed (three lines from patient 1 and one from patient 2; data not shown). This absence of staining is the same as that observed in 143B206, which completely lacks detectable COX activity (22). The wild-type lines all had similar, high levels of staining that were identical to that of 143B.

Selected cybrid cell lines derived from each patient were analyzed for their growth properties under several conditions. As shown previously (22), human cell lines that lack mtDNA require both uridine and pyruvate for cell growth and undergo less than one population doubling in the absence of either metabolite (Fig. 5A). Cybrid cell lines containing wild-type mtDNA, such as WS239 and RN236, grow as well in the absence of uridine or of pyruvate or of both uridine and pyruvate as in complete medium (Fig. 5C and E). These are the same characteristics of growth that have been found in other transmitochondrial cell lines (22). However, the growth properties of the cybrids with the MELAS mutation are strikingly different. In the absence of pyruvate, clones WS176 and RN164, and to a lesser extent WS240 (Fig. 5B, D, and F), show an initial lag in their rates of growth and do not reach the same density of cells per plate as do cells grown in the presence of pyruvate. In addition, their maximal rates of growth in the absence of pyruvate compared with growth in the presence of pyruvate were decreased approximately 10% for WS176 and WS240 and about 30% for RN164. The changes in the growth properties in the absence of pyruvate correlated with the rates of oxygen consumption in these cell lines, underscoring the requirement for a fully functional respiratory chain for growth in the absence of pyruvate. The presence or absence of uridine did not affect the growth characteristics of these mutant cell lines. The population doubling times of the mutant cell lines grown in the presence of pyruvate were not significantly different from those of the respective wild-type cell lines.

RNA analysis. Total RNA was extracted from exponentially growing cells of representative mutant and wild-type cell lines and examined by RNA transfer hybridization analysis (Fig. 6). The RNA was hybridized with probes derived from four mtDNA genes (12S rRNA, 16S rRNA, ND 1, and ND 5) as well as with a probe for nuclear-encoded cytoplasmic β-actin. The probes were hybridized sequentially after stripping off each previously used probe. In other RNA transfer hybridization analyses, in addition to these probes, mtDNA probes specifying the genes for COX II, $tRNA^{Leu(UUR)}$, and the light-strand 7S RNA transcript were used (data not shown). In all cases, no difference in the size of any transcript was observed between cybrids containing wild-type or mutant mtDNAs. However, in addition to the expected RNA species, one novel RNA transcript, not previously described, was identified. This RNA molecule corresponded to the 16S rRNA + $tRNA^{Leu(UUR)}$ + ND 1 genes (hereafter referred to as RNA 19, by extension of the nomenclature for the human mitochondrial RNA species [1]) and was identified on the basis of its hybridization to probes specifying each of its components, as well as by a comparison of its expected size (2,592 bases) with its apparent size as indicated by its electrophoretic mobility (2.9 kb). This was also the only RNA species that hybridized with the $tRNA^{Leu(UUR)}$ probe (tRNA molecules were not resolved by this system of gel electrophoresis).

The hybridization signals shown in Fig. 6 were quantitated; the averages of the ratios of the various species analyzed for the mutant and wild-type cell lines, as well as for the 143B cell line, are shown in Table 2. Except for ratios involving solely RNA 19 and ND 1 mRNA, which were quantified in the same hybridization analysis, the ratios are relative values and do not reflect the absolute numbers of each species. With the exception of RNA 19 (discussed below), no quantitative differences in the intensity of the hybridizing signal between mutant and wild-type cybrid cell lines were observed, including that for COX II. The ratios of each of the mitochondrial RNA species to β-actin were not significantly different between the mutant and the wild-type cybrid cell lines (Table 2). Assuming that the steady-state

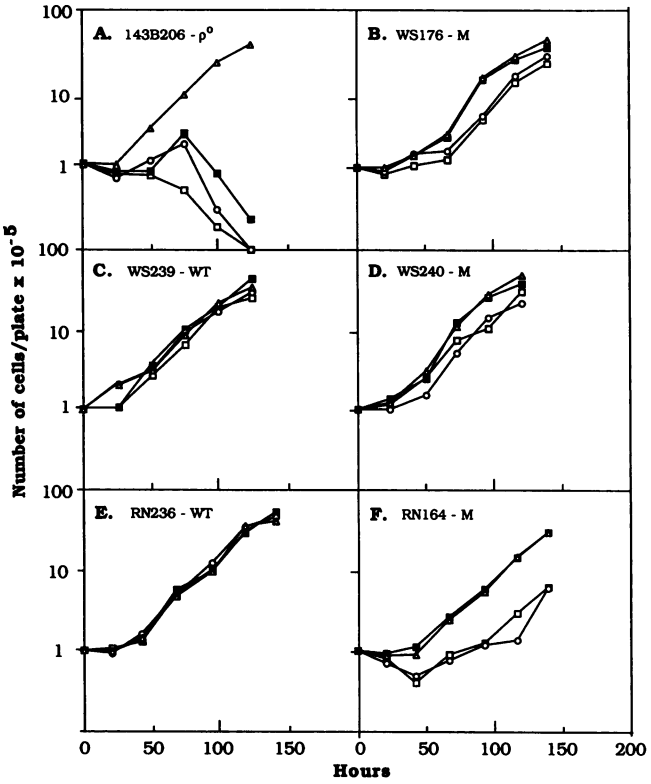

FIG. 5. Growth curves for 143B206 cells (A), wild-type (WT) cybrid cell lines WS239 and RN236 (C and E), and mutant (M) cybrid cell lines WS176, WS240, and RN164 (B, D, and F) in the presence or absence of uridine and pyruvate. Growth curves of the recipient ρ^0 cells and the cybrid cell lines were determined by seeding multiple 100-mm plastic petri dishes with a constant number of cells in 10 ml of DMEM supplemented with 5% dialyzed FBS plus 100 μg of BrdU per ml (open squares), the same medium supplemented with 50 μg of uridine per ml (open circles), the same medium supplemented with 110 μg of pyruvate per ml (solid squares), or the same medium supplemented with both 50 μg of uridine per ml and 110 μg of pyruvate per ml (open triangles). At various time intervals, cells from individual plates were trypsinized and counted.

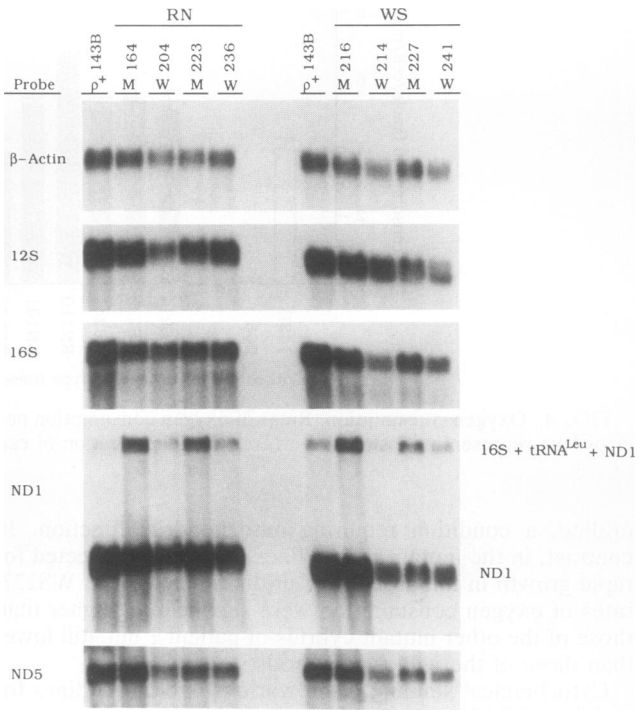

FIG. 6. Northern (RNA) blot hybridization analyses. Shown are autoradiograms of transfer hybridizations of total RNA isolated from exponentially growing 143B cells and the indicated mutant (M) and wild-type (W) cybrid cell lines hybridized with probes for β-actin, 12S rRNA, 16S rRNA, ND 1, and ND 5. The hybridization probe used is indicated at the left of each autoradiogram; the two species hybridizing to the ND 1 probe are indicated at the right. RNA 19 was also detected with the 16S rRNA probe but was not visible at the exposure time used to display mature 16S rRNA; the region of the autoradiogram containing RNA 19 is not shown.

levels of β-actin are not different between the cell lines, these results demonstrate that the amount of each mitochondrial RNA species per cell was unchanged in the mutant compared with the wild-type lines. In 143B, the ratios of ND 1 to β-actin and of ND 5 to β-actin were slightly higher than in the wild-type cell lines but were not significantly different from the ratios found in the mutant cell lines. The ratios of each of the mRNA species to both 12S and 16S rRNAs were nearly identical among all of the cell lines examined. This finding indicates that the levels of mRNAs in the mutant cybrids, which were regulated either transcriptionally or posttranscriptionally, were similar to those found in the wild-type cell lines.

The only quantitative difference that was observed between mutant and wild-type cell lines was a consistent increase in the levels of RNA 19 in cybrids containing the MELAS-3243 mutation compared with those containing wild-type mtDNA. In the hybridization analysis using the ND 1 probe (Fig. 6), the average RNA 19 level was 15% of that of the mature ND 1 transcript in the four wild-type cybrids examined but was 28% of the ND 1 level in the four mutant cybrids, an increase of 87% (Table 2). With use of the probe for 16S rRNA, the amount of RNA 19 was extremely low relative to that of mature 16S rRNA, which is an abundant transcript (RNA 19 was less than 2% of 16S rRNA). Because of this, it was impossible to quantitate accurately the differences in levels between wild-type and mutant cell lines with use of this probe. Significant increases were also observed between the mutant and wild-type cell lines when we compared the ratio of the RNA 19 signal to the signals of the other RNA transcripts. The increase ranged from an average of 58% for RNA 19/ND 5 to 98% for RNA 19/β-actin. Interestingly, the levels of RNA 19 were significantly higher in the wild-type cybrids of both patients than in the 143B cell line. For example, the level of RNA 19 was 7% of that of ND 1 in 143B but was 15% of the ND 1 level in the wild-type cybrids. The reason for this difference is unknown but may be due to differences in the mtDNA genotypes in these cells.

DISCUSSION

The results presented here demonstrate a direct correlation between the presence of the A→G transition at nt 3243 of the mitochondrial genome and a severe protein synthesis and respiratory chain defect in cells harboring this mutation. The fact that the same mutation, present in two different mtDNA genotypes, results in the same deficiencies of mitochondrial translation and respiration strongly suggests that this mutation alone is sufficient to produce the phenotypic

TABLE 2. Quantitation of hybridizing bands shown in Fig. 6[a]

Transcripts	Ratio (mean ± SD)			Ratio, M/W
	143B	M	W	
RNA 19/ND 1	0.07 ± 0.04	0.28 ± 0.10	0.15 ± 0.02	1.87
RNA 19/ND 5	0.36 ± 0.20	1.50 ± 0.34	0.95 ± 0.06	1.58
RNA 19/12S rRNA	0.16 ± 0.11	0.73 ± 0.14	0.43 ± 0.23	1.70
RNA 19/16S rRNA	0.48 ± 0.11	2.72 ± 0.42	1.56 ± 0.27	1.74
RNA 19/β-actin	0.28 ± 0.17	0.95 ± 0.26	0.48 ± 0.08	1.98
ND 1/β-actin	0.42 ± 0.02	0.37 ± 0.15	0.32 ± 0.03	1.16
ND 5/β-actin	0.76 ± 0.05	0.64 ± 0.14	0.50 ± 0.05	1.28
12S rRNA/β-actin	1.78 ± 0.18	1.33 ± 0.38	1.29 ± 0.48	1.03
16S rRNA/β-actin	0.55 ± 0.23	0.35 ± 0.08	0.30 ± 0.01	1.17
12S rRNA/16S rRNA	0.36 ± 0.18	0.39 ± 0.13	0.43 ± 0.16	0.91
ND 1/16S rRNA	0.85 ± 0.38	1.06 ± 0.40	1.05 ± 0.08	1.01
ND 5/16S rRNA	1.47 ± 0.51	1.90 ± 0.67	1.63 ± 0.20	1.17
ND 1/12S rRNA	2.40 ± 0.13	2.70 ± 0.53	2.80 ± 1.21	0.96
ND 5/12S rRNA	0.43 ± 0.07	0.50 ± 0.08	0.44 ± 0.21	1.14

[a] M, mutant; W, wild type.

effects of the MELAS syndrome. However, one cannot exclude the possibility that the phenotypic expression of the defect is due to one or more unidentified mtDNA mutations, acting in concert with the known MELAS mutation.

The most striking findings in this study were the marked decreases in both the rates of synthesis and the steady-state levels of the mitochondrial translation products. Presumably, the reduced rate of synthesis of mitochondrial proteins results in a decrease in the steady-state number of enzyme complexes and a dramatic decrease in mitochondrial respiratory chain activity. This alteration in the steady-state levels of the mitochondrial translation products does not appear to affect the synthesis or importation of nuclear-encoded mitochondrially located proteins, such as COX IV and PDH. The immunocytochemical staining for these polypeptides showed no change in the relative abundance or cytoplasmic distribution and localization between cells with or without the MELAS mutation, suggesting that both the numbers of mitochondria and their intracellular distribution are not altered in cells harboring the mutation.

While it is clear that this tRNA mutation is associated with a respiratory chain defect, the exact molecular mechanism by which this occurs was not established by the experiments reported here. Inhibition of translation due to a mutated tRNA gene may occur at several levels. The A→G transition at nt 3243, which is located at position 14 of $tRNA^{Leu(UUR)}$, may directly affect the mitochondrial tRNA function in translation by (i) inhibiting proper -CCA addition at the 3′ end of the tRNA (-CCA is added posttranscriptionally to the human mitochondrial tRNAs), (ii) affecting the recognition of the tRNA by enzymes involved in posttranscriptional modification of the tRNA, (iii) affecting recognition of the tRNA by the cognate leucyl-tRNA synthetase, which may lead to mischarging or noncharging of the tRNA, or (iv) inhibiting the correct recognition of the tRNA by translational elongation factors or by the ribosome.

The A at position 14 of the tRNA is virtually invariant among cytoplasmic tRNAs but does vary in some mitochondrial tRNA species (38). According to the X-ray crystal structure analysis of yeast $tRNA^{Phe}$, A-14 hydrogen bonds with U-8, and this base pairing is postulated to contribute to the stabilization of the tertiary structure of the tRNA (20, 24). If the tertiary structure is disrupted, a decreased metabolic stability of the tRNA might be expected. However, no significant changes in the steady-state levels of $tRNA^{Leu(UUR)}$ have been observed (23).

Surprisingly, there was no correlation between either the number of Leu(UUR) codons or the fraction of Leu(UUR) codons present in the mitochondrial genes and the apparent rate of labeling of the corresponding polypeptides in the mutant cybrids. In fact, the polypeptide with the largest number of Leu(UUR) codons, ND 6, showed no reduction in labeling. This and the fact that the translation pattern is qualitatively normal in the mutant cybrids (although quantitatively decreased) suggest that -CCA addition and tRNA charging with leucine do occur. This result contrasts with what has been found in MERRF cybrids, in which the number of Lys codons correlates with a reduction in the labeling of mitochondrial polypeptides (6). Thus, the pathogenetic mechanisms in the two diseases are likely to be quite different. The absence of a decrease in the labeling of ND 6, the only light-strand-encoded polypeptide, suggests that the defect in MELAS might be specific for polypeptides encoded by the heavy strand.

In vitro, this mutation has been shown to decrease dramatically the proper termination of transcription at the end of the rRNA genes (17). A termination error might be expected to lead to altered ratios of rRNAs and mRNAs, since the rRNA genes are transcribed at a rate 15- to 20-fold higher than the rate for most of the protein-coding genes (11). However, we observed no significant differences in the steady-state levels or in the ratios of RNAs representative of the two heavy-strand transcription units, nor were the amounts of heavy-strand transcripts altered significantly in relation to the 7S RNA light-strand transcript. However, because 7S RNA does not encode a polypeptide, it may not be representative of the light-strand transcripts coding for ND 6. Analysis of muscle biopsies from MELAS patients by in situ hybridization using probes specific for both heavy- and light-strand transcripts was consistent with the results presented here (31). This finding strongly suggests that an absence of specific termination at the end of the rRNA genes is not the primary mechanism by which the MELAS-3243 mutation causes the respiratory-deficient phenotype. However, our experiments did not address whether the proper steady-state levels of the various RNA species were controlled transcriptionally or posttranscriptionally. Since posttranscriptional regulation is an important method of controlling the steady-state levels of the mitochondrial RNAs (11, 21), we cannot exclude the possibility that the levels of termination of transcription at the end of the rRNA genes were decreased in vivo when the MELAS-3243 mutation was present, but that the correct levels of rRNAs and mRNAs were maintained by a more rapid turnover of the mRNAs.

Another possible effect of the MELAS point mutation is an alteration in the processing of the large polycistronic transcript of the heavy strand of the mtDNA. Presumably, the tRNA sequences, such as $tRNA^{Leu(UUR)}$, which flank nearly every rRNA and protein-coding gene, serve as recognition signals for those enzymes which make the precise endonucleolytic cleavages at the 5′ and 3′ ends of the tRNAs, thereby excising the tRNAs and releasing the rRNAs and the mRNAs. If this precise recognition of the $tRNA^{Leu(UUR)}$ is altered by the MELAS mutation, then processing of the primary transcript may be inaccurate, may not occur at all, or may occur precisely but at a reduced rate. This could alter, either qualitatively or quantitatively, not only the $tRNA^{Leu(UUR)}$ but also the 16S rRNA and ND 1 mRNA which flank this tRNA in the primary transcript. It is

clear from the increase in the steady-state levels of the RNA species corresponding to the 16S rRNA + $tRNA^{Leu(UUR)}$ + ND 1 genes (i.e., RNA 19) that the processing at the 5′ and 3′ ends of $tRNA^{Leu(UUR)}$ does not occur in some transcripts or occurs at a reduced rate in the mutant cybrids.

In RNA transfer hybridization experiments with a probe corresponding to $tRNA^{Leu(UUR)}$, we found no evidence of an RNA corresponding to the $tRNA^{Leu(UUR)}$ + ND 1 genes (i.e., processing activity only at the 5′ end of the tRNA) or the $tRNA^{Leu(UUR)}$ + 16S rRNA genes (i.e., processing only at the 3′ end). Combined with the elevated levels of RNA 19 in the mutant cybrids, this finding strongly suggests that there is an inhibition of the 5′ and 3′ processing activities. These activities may act in concert or, alternatively, there may be an obligate order of cleavage, in which the enzyme that cleaves first (either the 5′ or the 3′ endonuclease) is rate limiting.

The RNA transfer hybridizations did not reveal any other large-scale alterations in the processing of the polycistronic transcript, but smaller errors of a few nucleotides would not be detected by the methodologies used in this work and cannot be excluded. Suggestive of such a small alteration in processing is the increase in electrophoretic mobility observed for ND 1. If the ND 1 mRNA is processed inaccurately at its 5′ end in such a way that the first AUG codon (beginning at nucleotide +3 of the wild-type mRNA) is removed, translation of ND 1 may begin at one of the internal AUGs (e.g., at in-frame codon 3, 17, or 21), resulting in a polypeptide that is shorter than the normal protein. Such an alteration in ND 1 also agrees with the observations that in the majority of clinically defined MELAS cases, there is a disproportionate deficiency of complex I relative to the other complexes (13, 26).

The relatively small steady-state levels of RNA 19 relative to the mature products, and the fact that the steady-state levels of ND 1 and 16S rRNAs are unaffected, might suggest that the elevated levels of RNA 19 are not the key mechanism by which the MELAS-3243 mutation results in the large alterations in mitochondrial translation and respiration reported here. The fact that levels of RNA 19 are twofold higher in the wild-type cybrids than in 143B cells yet both groups have nearly identical levels of cellular respiration also suggests that increased levels of this RNA species have no obvious phenotypic consequence. However, there is a very strong inverse correlation between the levels of RNA 19 per cell (i.e., RNA 19/β-actin) and the rate of oxygen consumption of the cybrid cell lines (data not shown), suggesting that this molecule may indeed have functional consequences. RNA 19 could be directly responsible for the observed inhibition of protein synthesis, or it may merely reflect other errors that are also occurring in the mutant cybrids. Since RNA 19 contains 16S rRNA, this transcript could be incorporated into ribosomes, rendering them functionally deficient. If such an alteration resulted in ribosome stalling on polyribosomal mRNAs, even low levels of RNA 19 could be responsible for such a dramatic effect on mitochondrial translation. This view is also consistent with the fact that the larger mitochondrial polypeptides appear to be disproportionately inhibited in their synthesis compared with that of the smaller polypeptides. This mode of inhibition of synthesis of larger polypeptides would also be consistent with alteration of the 3′ end of the 16S rRNA caused by misprocessing or by premature transcription termination.

It may well be that the single qualitative alteration in the genotype, the A→G mutation in the $tRNA^{Leu(UUR)}$ gene, is pleiotropic, causing a number of small but distinct effects, each of which contributes in a quantitative way to the overall reduction in mitochondrial function documented here. These cumulative effects would include (i) altered processing of the primary transcript, (ii) increased levels of the transcript corresponding to the 16S + $tRNA^{Leu(UUR)}$ + ND 1 genes (RNA 19), (iii) decreased termination of rRNA transcripts, (iv) expression of an aberrant ND 1 polypeptide, (v) altered kinetics of leucyl-tRNA aminoacylation, and (vi) noncharging or mischarging of some $tRNA^{Leu(UUR)}$ molecules. The combination of these specific factors could produce the MELAS phenotype.

MERRF is also associated with a mtDNA point mutation in a tRNA gene. It has been shown that the MERRF mutation causes a decrease in the rate of mitochondrial translation and a deficiency in cellular respiration (6), similar to what has been described here in MELAS. Despite these similarities, MERRF and MELAS are clinically distinct and distinguishable entities. Thus, it may be that small alterations in normal mitochondrial function specific to each mutation lead to the distinct clinical phenotypes.

The identification of point mutations of mtDNA which lead to human disease has been based primarily on the sequencing of all or specific portions of the mitochondrial genome of patients with mitochondrial disease and the comparison of these sequences with those of other human mtDNAs and with the mtDNAs of other species. Unfortunately, these approaches do not address the etiology and pathogenesis of these diseases. Through the use of human cell lines which lack mtDNA and the ability to repopulate these cells with exogenous mitochondria, it is possible to examine the phenotypic consequences of these point mutations at the cellular and molecular levels, aiding in the identification of the molecular defects.

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